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INVITRO CYTOTOXIC ACTIVITY OF GLYCOSIDES FROM LEAVES AND SEEDS OF CORCHORUS SPECIES AGAINST K-562 AND VERO CELL LINES

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ABSTRACT

The study was aimed to evaluation of the antileukaemic activity of the leaves and seeds of *Corchorus aestuans* L., *Corchorus olitorius* L. and *Corchorus trilocularis* L. on the K-562 cell line. The methanolic extract of Corchorus species have been screened for antileukaemic potentials using in vitro assay for Cytotoxic activity (MTT assay) with ten different concentrations. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The percentage viability of the cell line was carried out by using Trypan blue dye exclusion method. The concentration causing 50% cell growth inhibition (IC_{50}) was determined from DRC using Graph Pad Prism software. *Corchorus aestuans* L. seed (IC_{50} value 37.09µg/ml, R^2=0.9930) and *Corchorus trilocularis* L. leaf (IC_{50} value 67.44µg/ml, R^2=0.9952) showed good cytotoxicity activity as compare to Std. drug (Colchicin) having IC_{50} value 105.5 µg/ml, R^2=0.9950).

KEYWORDS: Cytotoxicity, K-562, *Corchorus aestuans* L., *Corchorus olitorius* L., *Corchorus trilocularis* L.

INTRODUCTION

Cancer is still a serious health problem and has a major social and economic impact worldwide. Despite recent advances in diagnosis, prevention, and therapy, cancer still affects quality of life in patients due to some limitations of these current medical practices. Consequently, more and more people resort to alternative medicine, which is defined as health care practices used instead of standard ones. Herbal medicine, one type of the alternative medicine, is based on the use of plants or plant extracts to treat diseases and promote health and has been offered especially for cancer treatment over the last century. This alternative treatment is more widely accepted at the present time. Therefore, medicinal plants have become important and reliable sources for anticancer agents and worldwide efforts are ongoing to find new plants with biological activity. Natural products are known to provide lead compounds in the past and play a significant role in future in the treatment of cancer.[1-4]

Corchorus is a genus of about 40-100 species of flowering plants in the family Tiliaceae, native to tropical and subtropical regions throughout the world.[5]

Different common names are used in different contexts, with jute applying to the fiber produced from the plant, and mallow-leaves for the leaves used as a vegetable. The English common name is 'mallow'.[6]

Corchorus species are used as diuretic, chronic cystitis, gonorrhoea, dysuria, antihistaminic, anti-inflammatory, antimicrobial, cardiotonic, and also to increase the viscosity of the seminal fluid.[7-8]

Specific cells that can grow indefinitely given the appropriate medium and conditions i.e. living cells those are maintained in-vitro in artificial media of serum and nutrients for the study and growth of certain strains, experiments in controlling diseases, or study of the reaction to certain drugs or agents. Human tumor cell line panels combined with rapid high throughput cytotoxicity testing have proven to be valuable tools for drug screening and early drug evaluation and investigation of drug resistance mechanisms. The Vero cell line was initiated from the kidney of a normal adult African green monkey on March 27, 1962, by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan. The continuous cell line K-562 was established by Lozzio and Lozzio from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises.[9]

MATERIALS AND METHODS

Materials

Plant material collection

Fresh & fully grown plants of *Corchorus aestuans* L., *Corchorus olitorius* L. and *Corchorus trilocularis* L. were collected from nadiad in the month of September and their authentification were confirmed by Dr. A.M. Patel, Dept. of Botany, J & J College of science, Nadiad.

Reagents
Trypan blue Dye (Hyclone, Lot No: 029K2358), Triton X100 (MP Biomedicals, Lot No: 8009H), DMSO cell culture grade (Bioworld, Lot No: 1388B230), Sodium bicarbonate (Bioworld, Lot No: 1775B29), Amphotericin B (Himedia, Lot No: 1397893), Penicillin and Streptomycin solution stabilized (Sigma, Lot No: 1208029), EDTA (MP Biomedicals, Lot No: YY02022B207Y), DPBS / modified 1X (Dulbecoo's phosphate buffer saline without Ca^{+} and Mg^{+}) (Himedia, Lot No: LW537), Trypsin 1X Gamma irradiated (SAFC Bioscience, Lot No: 8NO535), Methotrexate (MP Biomedicals, Lot no. R27204), Triton – X 100 (Bioworld, Lot no. 1 8278075), Iso Propanol (Finar Chemicals, Lot no. 19075330).

Cell proliferation kit
MTT (Roche applied sciences, Cat. No. 465 007 001), MTT Dye Powder (Serva Electrophoresis, Lot no. 080364)

Media
DMEM (Dulbecoos Modified Eagels medium, low glucose with glutamine) (MP Biomedical, Lot No: C1478), FBS (Fetal Bovine Serum, South American origin, 500 ml) (Quaditive, Lot No: 103128), SFM HEK^{TM} (Hyclone, Lot no: ARF26635), Fluid thioglycolate media (TGM) (Himedia, Lot No: YHI25), Tryptone Soya broth (TSB) (Himedia, Lot No: YH031).

Glass wares and plastic wares
96-well microtiter plate (Flat Bottom, U Bottom, V Bottom), Tissue culture flasks (75 cm^2 T Flask vented and 150 cm^2 T Flask vented), Falcon tubes (15 ml, 50 ml), Cryotubes (2ml), Cell scrapper, Reagent bottles (100 ml, 250 ml, 500 ml, 1000 ml), Micro tips (Blue 1000 µl, Yellow 200 µl, White 10 µl) (Volex), Haemocytometer cell counting chamber.

Equipments
Fluorescence inverted microscope (Leica DM IL, Germany), Biosafety cabinet classII (Esco, Singapore), Cytotoxic safety cabinet (Esco, Singapore), CO_2 incubator (RS Biotech, mini galaxy A, Scotland), Deep freezer (Dairei, Denmark), ELISA plate reader (Thermo, USA), Micropipettes (Eppendorff, Germany), RO water system (Millipore, USA), Electronic water bath (Genei, India), Gel Electrophoresis Assembly, Transilluminator (Genei), Gel Doc Syatem (Genei), Shaker Incubator (Genei. India), Minispin Centrifuge (Eppendorff, Germany), Colony Counter, Vortex Shaker (Genei. India), Inverted Electronic Microscope (Leica)

Methods

- **Preparation of Plant extracts**

Soxhlet extraction was performed using classical soxhlet apparatus with accurately weighed 10g of leaf powder as well as seed powder (screened through mesh 22) for 14hr. Extraction was performed with 250 ml of methanol as well as solvent ether as the extracting solvents. Finally extracts were evaporated to dryness and DMSO was added into the dry extracts to achieve concentration of 1000 µg/ml.

Phytochemical Screening
Test for Steroids and Triterpenoids.[10-11]
Liebermann –Burchard reaction : Mix 2ml extract with chloroform .Add 1-2 ml aceteic anhydride and 2 drops conc. H2SO4 from the side of test tube. First red, then blue and finally green color appears.

Test for Saponins.[12-13]
Foam test: shake the drug extract or dry powder vigorously with water. Persistent foam observed.

Test for Flavonoids.[14]
Shinoda test: To dry powder or extract, add 5ml 95%ethanol, few drops conc. HCl and .5gms magnesium turnings, Pink color observed.

Tests for Cardiac glycosides.[15]
Baljet's test: A thick section shows yellow to orange color with sodium picrate.

Legal's test: To aqueous or alcoholic extract, add 1 ml. pyridine and 1ml. sodium nitroprusside. Pink to red color appears.
Keller-Killiani test: To 2 ml. extract, add glacial acetic acid, one drop 5% Ferric chloride and conc. Sulphuric acid. Reddish brown Color appears at junction of the two liquid layers and upper layer appears bluish green.

- **Testing for Microbial Contamination**[16]

The two methods generally used by us in our laboratory to check for bacterial and fungal contamination. Detection carried out using special media like Fluid thiogycolate media (TGM) and Tryptone Soya broth (TSB) and direct observation using Grams stain.
Contamination by bacteria, yeast or fungi was detected by an increase in turbidity of the medium and/or a decrease in pH (yellow in media containing phenol red as a pH indicator). Cells were inspected daily for presence or absence of microbial growth.

Protocol.[17]
Cell lines were cultured in the absence of antibiotics prior to testing using 25 cm^2 non-vented T-flask.

In case of adherent cell line, attached cells were bringing into suspension using a cell scraper. Suspension cell lines were tested directly. 1.5 ml test sample (Cells) were Inoculated in to two separate test tubes of each containing Thioglycollate Medium (TGM) and Tryptone Soya broth (TSB). 0.1 ml E. Coli, 0.1 ml B. Subtilis and 0.1 ml C. Sporogenes inoculated in to separate test tubes (duplicate) containing (TGM) and (TSB). These were act as positive controls where as two separate test tubes of each containing (TGM) and (TSB) un-inoculated as negative controls.

Broths were incubated as follows

- For TSB, one broth of each pair were incubated at 32 ºC the other at 22 ºC for 4 days.
- For TGM, one broth of each pair were incubated at 32 ºC the other at 22 ºC for 4 days.
- For the TGM inoculated with C. Sporogenes incubate at 32 ºC for 4 days.

Note: Test and Control broths were examined for turbidity after 4 days.

Design of experiment

Cell lines in exponential growth phase were washed, trypsinized and re-suspended in complete culture media. Cells were seeded at 2×10^4 cells / well in 96 well microtitre plate and incubated for 24 hrs during which a partial monolayer forms. The cells were then exposed to various concentrations of the test compounds (as indicated in plate assignment) and standard doxorubicin. Control wells were received only maintenance medium. The plates were incubated at 37 °C in a humidified incubator with 5 % CO_2, 75 % Relative Humidity for a period of 24 hrs. Morphological changes of drug treated cells were examined using an inverted microscope at different time intervals and compared with the cells serving as control. At the end of 24 hrs, cellular viability was determined using MTT assay.[18]

Screening of test compound by MTT assay [19,20]:

- Cells were preincubated at a concentration of 1×10^6 cells / ml in culture medium for 3 hrs at 37°C and 6.5 % CO2, 75 % Relative Humidity.
- Cells were seeded at a concentration of 5×10^4 cells / well in 100 µl culture medium and various amounts of compound (final concentration e.g. 1000 µg/ml – 0.05 µg/ml) were added into microplates (tissue culture grade, 96 wells, flat bottom).
- Cell cultures were incubated for 24 hrs at 37 °C and 6.5% CO2.
- 10 µl MTT labeling mixture was added and incubate f or 4 hrs at 37 °C and 6.5 % CO2, 75 % Relative Humidity.
- 100 µl of solubilization solution was added to each well and incubate for overnight.
- Absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 540 and 600 nm according to the filters available for the ELISA reader, used. (The reference wavelength should be more than 650 nm).

RESULTS

Phytochemical Screening

Qualitative chemical examination of methanolic extracts of leaf and seed showed the presence of steroids, triterpenoid glycosides, flavonoid glycoside and cardiac glycoside.

Characterization of cell lines and culture media

Characterization of cell lines was performed for detection of microbial and cross contamination. Cell lines used in our experiments were free from any kind of microbial or fungal contamination (Table No. 1), which in essential in order to continue our screening experiments.

Culture media were also tested for microbial contaminations. To prevent microbial contamination, 2.5 % Amphotericin B (µg/ml) was supplemented to media which act as working concentration. Bacterial contamination was prevented by addition of 1 % of Antibiotic, 100 X (10000 U/ml Penicillin G, 10000 µ g/ml Streptomycin) into culture medial. All subculturing activities were done under class – II Biosafety cabinet. (Esco, Singapore).

Cross contamination of cell line was tested by direct observation of particular cell line under inverted microscope. PDT for specific cell line was determined. From viability studies and PDT, we have concluded that the cell lines derived from ATCC were initially free from cross contamination.

To prevent the cross contamination of cell lines during our experiments work, separate pipettes and plastic tips were used for individual cell line. Along with that, particular cell line was used at the time under Class – II Bio safety cabinet. These were proving to be valid steps to prevent cross contamination of cell lines throughout the experiment (Table 1).

Cell viability, density and population doubling time

VERO cell line

At the time of Subculture, Vero cell line was having viability 54.54%,, which was not significant as per protocol to perform cytotoxicity study. In order to increase the viability and cell density of VERO cell line, subculturing was done by using complete media and additional 5% FBS along with BSS. As a result, on the fourth day morning viable cell density was increased up to 46×10^7 and viability was around 71.91% which was suitable for cytotoxicity screening. PDT= population doubling time for VERO was 27.9hr. (Table No. 2) represents Result for Subculture of VERO cell.

PDT of 27.9hr indicates that population of cells were double after every 27.9 hrs. As the population of cells in the flask increase, more amounts of media were consumed by cells for growth purpose and this lead to acidic pH of the media, which requires continuous addition of media for maintenance of pH and nutritional requirements. Subculturing was performed every 3rd or 4th day i.e. twice in week.

K-562 cell line

At the time of Subculture, viability of K-562 cell line was around 58.61 %, which was not suitable for cytotoxicity study, considering requirement of cell viability greater than 80 %. In order to increase the

viability and cell density of K-562 cell line, subculturing was done by using complete media and additional 5 % FBS. As a result, on the fourth day morning cell density was increased and viability was around 87.98 % which was suitable for cytotoxicity screening. PDT = Population Doubling Time for K-562 was 19.23 hrs. (Table No. 3) represents result for Subculture of K-562 cell.

Average PDT for K-562 was found to be 19.23 hrs. As the population of cells in the flask increase, more amounts of media were consumed by cells for growth purpose and this lead to acidic pH of the media, which requires continuous addition of media for maintenance of pH and nutritional requirements. Subculturing was performed every 3rd or 4th day i.e. twice in week.

Activity of various extracts on K-562 cell line by MTT assay

Serially diluted extracts of leaves and seeds in DMSO was incubated with the K-562 cell line for 24 hrs then MTT dye was added. Absorbance was taken after adding solubilizing agent at 540 nm wavelength in the ELISA plate reader. The data were analysed with graphpad prism, sigmoidal dose response (Fig. 1) and IC_{50} value was determined (Table No. 4).

Table 1: Results for Characterization of cell lines

Cell line	%viability		PDT(hrs)	Microbial Contamination	Cross Contamination	PH
	Stock	After				
VERO	54.54	71.91	27.9	No contamination	No	7.5
K-562	58.64	73.9	19.23	No contamination	No	7.5

Table 2: Result for Subculture of VERO cell line

Days	1st	2nd	3rd	4th
Viable cell count	33	43	67	89
Non-viable cell count	18	27	39	34
% viability	54.54	62.79	67.16	71.91
Cells/ml	3.6×10^{5}	5.4×10^{5}	9×10^{5}	12.8×10^{5}
Viable cells in flask(50ml)	9.81×10^{7}	16.95×10^{7}	30.2×10^{7}	46×10^{7}
PH	7.0	5.0	4.5	5.0
PDT				27.9 hrs

Table 3: Result for Subculture of K-562 cell line

Days	1st	2nd	3rd	4th
Viable cell count	37	43	70	77
Non-viable cell count	21	24	17	11
% viability	58.61	60.79	80.16	87.98
Cells/ml	3.6×10^{5}	5.4×10^{5}	9×10^{5}	12.8×10^{5}
Viable cells in flask(50ml)	1.8×10^{7}	2.7×10^{7}	4.5×10^{6}	6.1×10^{7}
PH	7.5	5.0	4.5	5.0
PDT				19.23 hrs

Table 4: % Cell inhibition by various extract on K-562 cell line.

Conc. µg/ml	Log con.	% Cell Inhibition						
		Sample-1: C.ast leaf	Sample-2: C.olt leaf	Sample-3: C.tri leaf	Sample-4: C.ast seed	Sample-5: C.olt seed	Sample-6: C.tri seed	Colchicine
0.05	-1.29	0.01	-0.20	0.17	-0.30	0.22	0.07	2.06
0.15	-0.82	0.15	0.01	1.12	0.55	0.78	0.03	2.44
0.46	-0.34	1.26	0.46	2.45	1.75	1.45	0.58	3.06
1.37	0.14	1.88	1.75	4.82	3.14	1.68	0.55	3.77
4.12	0.61	2.75	2.86	7.38	2.85	2.44	1.65	9.25
12.35	1.09	7.02	87.55	15.46	14.32	5.36	3.35	14.77
37.04	1.57	14.21	14.72	26.85	22.61	7.15	15.82	22.46
111.11	2.05	28.03	26.88	47.77	36.52	12.45	26.75	40.04
333.33	2.52	40.21	28.75	58.68	40.42	15.52	45.39	58.60
1000	3.00	67.35	61.57	71.93	47.12	36.12	66.14	70.49
IC_{50} (µg/ml)		**280.5**	**1139**	**67.44**	**37.39**	**1075**	**221.2**	**105.5**
R^2		**0.9861**	**0.2436**	**0.9952**	**0.9930**	**0.9616**	**0.9948**	**0.9950**

Table 5: %Cell inhibition by various extracts on Vero cell line.

Conc. µg/ml	Log con.	% Cell Inhibition						
		Sample-1: C.ast leaf	Sample-2: C.olt leaf	Sample-3: C.tri leaf	Sample-4: C.ast seed	Sample-5: C.olt seed	Sample-6: C.tri seed	Colchicine
0.05	-1.29	-0.38	-0.97	0.06	-0.12	0.03	-0.24	0.05
0.15	-0.82	0.05	-0.65	0.09	0.02	1.02	0.0	0.06
0.46	-0.34	0.43	0.07	0.0	0.65	1.46	0.56	0.65
1.37	0.14	1.01	1.02	0.68	0.79	2.04	0.98	0.97
4.12	0.61	1.23	1.64	2.03	1.07	2.64	1.06	1.06
12.35	1.09	2.01	2.06	2.46	3.56	3.05	2.03	1.68
37.04	1.57	2.43	6.34	3.03	5.31	7.21	2.56	2.31
111.11	2.05	3.02	15.24	5.12	11.32	16.34	3.45	3.04
333.33	2.52	4.23	32.21	10.34	24.31	18.31	4.35	11.34
1000	3.00	26.34	46.21	40.32	47.82	36.35	35.62	44.35
IC_{50} (µg/ml)		**>1000**	**811.5**	**>1000**	**∞1000**	**989.3**	**>1000**	**>1000**
R^2		**0.9562**	**0.9982**	**0.9883**	**0.9975**	**0.9608**	**0.9467**	**0.9898**

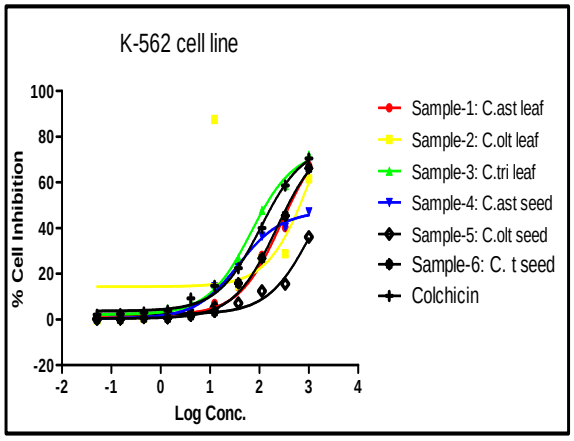

Figure 1: Dose response curve of various extract of leaves.

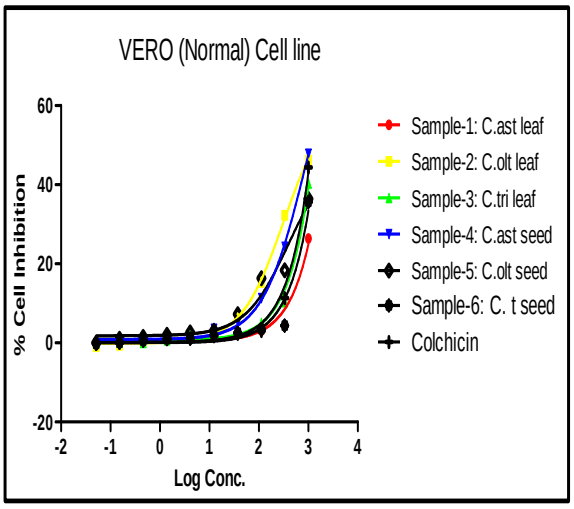

Figure 2: Dose response curve of various extract of leaves against Normal cell line by MTT assay method.

DISCUSSION

In the present work, all the extracts were evaluated against cell lines named K-562 and VERO. For each tested compound, Dose Response Curve (DRC) against cell lines was plotted with 10 analysis point i.e. with 10 different drug concentrations. The concentration causing 50% cell growth inhibition (IC_{50}) was determined from DRC using GraphPad Prism software (Ver. 5.04) (GraphPad Software, Inc., USA) and Micorsoft Excel 2007 (Microsoft Corporation, USA) application.

In K-562 cell line study Sample-4 (IC_{50} value 37.09µg/ml, R^2=0.9930) and sample-3 (IC_{50} value 67.44µg/ml, R^2=0.9952) showed good cytotoxicity activity as compare to Std. drug (Colchicin) having IC_{50} value 105.5 µg/ml, R^2=0.9950. Moreover, sample-6 and sample-1 had shown less activity while sample-5 and sample-2 did not give any activity. While performing cytotoxicity effect of test compounds against normal cell line i.e. VERO, most of compounds having IC_{50} value more than 1000 µg/ml which is safe as per FDA guidelines (Table No. 5, Fig 2).

CONCLUSION

Overall results shows that *Corchorus aestuans* L. seed and *Corchorus trilocularis* L. leaf having good cytotoxic effect on K-562 cell line Normal cell cytotoxicity study shows that all samples are safe to normal cell.

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